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Subnanomolar Detection of Acid-Labile Sulfides by the Classical Methylene Blue Method Coupled to HPLC

STEVEN E. MYLON* AND GABOURY BENOIT

Yale School of the Environment, Yale University, New Haven, Connecticut 06511

Methylene blue reactive sulfides (MBRS) are ubiquitous in surface waters of local lakes and streams. However, in most cases they exist in concentrations too low to measure by traditional methods. Both the classical methylene blue and the similar Cline method have been used to determine concentrations of a particular pool of acid-labile sulfides in aquatic systems. The estimated lowest limit of MBRS detection is ca. 10 nM under ideal conditions. By coupling the classical methylene blue method to HPLC, it is possible to make direct, reliable measurements of MBRS in fully oxygenated freshwaters. Using this modified approach, a detection limit for MBRS of ca. 0.3 nM is achieved. Measurements in a variety of local freshwaters reveal levels in the range of 0−74 nM, with systematic variations explainable in terms of standard biogeochemical characteristics.

Introduction

Historically, natural organic matter (NOM) has been implicated in speciation of dissolved heavy metals in aquatic systems (*1*−*4*). Hard−soft acid−base theory predicts preferential complexation of B-type metals to soft Lewis acids, good examples of which are reduced sulfur compounds (*5*, *6*). In fully oxygenated surface waters, however, metal complexation to reduced sulfur compounds must compete with the rapid oxidation of these sulfides to sulfate (*7*). In light of this, researchers have not sought to measure low levels of stable reduced sulfur compounds in freshwater systems, and attention has been focused on metal−NOM complexation.

Only recently have low steady-state concentrations of sulfides been observed in fully oxygenated freshwater systems (*8*−*11*). These small (ca. 2−10 nM) concentrations compare well with the levels at which many dissolved heavy metals are normally found, suggesting that metal sulfides contribute significantly in the speciation of trace metals. Furthermore, the association of some metals with reduced sulfur compounds may contribute to a decrease in rates of sulfide oxidation (*12*, *13*). The stabilizing effect on sulfides that these metals may contribute in oxygen-saturated environments raises the possibility of reduced sulfur as an important component in the partitioning of B-type metals in the environment. In fact, thermodynamic modeling done in this lab using metal concentrations typical of Connecticut surface waters suggests that much of the dissolved sulfides should be bound by Cu and Zn. Also, virtually all of the Cu and Ag and most of the Zn and Pb should be locked up as sulfide complexes over the normal range of pH. Thermodynamic data for sulfide complexes and precipitates are often unreliable, but this example illustrates the potential importance of interactions between dissolved reduced sulfides (DRS) and trace metals. Low levels of DRS might be able to outcompete more abundant NOM and bind metals if conditional stability constants are adequately high; therefore, they may be as important as NOM in the fate, transport, and bioavailability of these metals in lakes, rivers, and streams. In addition, even some of the metal binding by NOM may involve functional groups containing reduced sulfur.

Both the classical methylene blue and the Cline (*14*) methods have been used to determine only acid-labile or methylene blue reactive sulfide (MBRS) concentrations in aquatic systems. Among these MBRS are H_2S, HS^-, S^{2-}, FeS, ZnS, CdS, and MnS. Pyrite, CuS, and sulfide from organosulfur compounds are not included as MBRS, and only terminal polysulfides (S_nS^{2-}) are included as MBRS (*13*). These methods employ a two-step reaction in which solutions are acidified to strip sulfides from their associated compounds and convert them to dissolved H_2S. Under acidic conditions, the *N*,*N*-dimethyl-*p*-phenylenediamine is oxidized by Fe^{3+}, and the resulting product reacts with H_2S to form methylene blue (*15*). While these two methods differ with respect to types of acid, the counterion associated with the acids do not appear to have an affect on the yield of methylene blue. Standard solutions are diluted such that, within the measured concentration ranges, methylene blue strictly adheres to Beers law (*14*); therefore, acid-labile sulfide concentrations can be measured spectroscopically. A simple Beers law calculation yields an optical density of ca. 7.4×10^{-4} for a 1 nM MBRS solution in a cell with a 10 cm path length. In light of this calculation, this method appears to have a lower limit of MBRS detection of ca. 10 nM under ideal conditions. Naturally, this technique is also limited by the precision of spectrophotometers. Such low concentrations of MBRS and the need for high-precision instrumentation make it nearly impossible to directly measure MBRS concentrations in fully oxygenated freshwater systems using affordable commercial instruments.

Another obstacle to reliable MBRS measurements in oxygenated aquatic systems is interference from NOM at λ_{max}. Dissolved NOM contributes to the overall optical density of the methylene blue solution and is nearly impossible to properly account for during calibration. In a recent laboratory experiment, an effective extinction coefficient for NOM from the Hammonasset River in Connecticut was determined to be ca. 4×10^{-4} L cm^{-1} (mg of DOC)$^{-1}$. Clearly the contribution to the overall absorbance at λ_{max} from NOM can significantly interfere with methylene blue derived from waters where the sulfide concentration is low. For this reason, many reported concentrations of MBRS may have been overestimated.

In an attempt to investigate heavy metal speciation by MBRS in fully oxygenated surface waters, we have developed an extremely sensitive technique for measuring total MBRS by the classical methylene blue method coupled to HPLC to isolate and concentrate the dye. Tang and Satschi (*16*) have recently reported an average dissolved sulfide concentration of 4.3 ± 0.6 nM in Galveston Bay, TX, using a similar method of methylene blue separation on Sep-Pak plus tC_{18} columns. Both methods offer the advantages of low limits of detection that are required for studies of oxygenated surface waters. These methods also have the benefit of no interferences from

* Corresponding author phone: (203)432-9229; fax: (203)432-3929; e-mail: steven.mylon@yale.edu.

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NOM or other species that may contribute to the overall absorbance at λ_{max}. This work is not meant to be a comparison between the methods of Tang and Santschi and ours. They used a different HPLC column, buffer, and eluent gradient. They also measured local sulfide concentrations through a method of standard additions while we make standards and a corresponding standardization curve. Finally, their measurements are in seawater. We report here on a similar method that has improved detection limits and its application to a variety of freshwater systems as follows:

(i) Reconnaissance of MBRS in New Haven region streams. Some of those high in MBRS concentrations will be further investigated to determine in what compounds these sulfides exist.

(ii) High temporal resolution study of MBRS during lake mixing. Changes in MBRS concentrations in surface waters of a stratified lake (Linsley Pond, Branford, CT) were documented during lake mixing in the late fall.

(iii) High spatial resolution sampling of an urban stream. MBRS were measured moving downstream along Wintergreen Brook, a tributary to the West River in New Haven, CT.

Experimental Section

Stock solutions of the mixed diamine reagent (MDR) were prepared according to the method described by Cline (*14*) for measuring acid-labile sulfide concentrations in the range of 1−3 μM. In detail, 0.5 g of *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (Alfa Aesar 98%) and 0.75 g of $FeCl_3 \cdot H_2O$ (Baker reagent grade) were dissolved in a cool 50% (v/v) solution of HCl (reagent grade). The resulting solutions are stored in the dark under refrigeration for no more than 2 weeks.

For the purposes of calibration, stock solutions of a primary sulfide standard (1.0 g of S^{2-}/L) were prepared using solid $Na_2S \cdot 9H_2O$. The solid was rinsed free of oxidation products, dried, and then weighed. We dissolved the solid in Nanopure water that had been deoxygenated by vigorous bubbling with high-purity nitrogen for a minimum of 1 h, and we adjusted the pH to 10 by adding NaOH. Primary standards were kept under refrigeration for no more than 2 weeks. While loss of some sulfide due to adsorption onto borosilicate glass containers has been observed by others (*13*), numerous standardization curves over the course of these studies differed only within the reported limits of uncertainty. In light of this, we remained unconcerned with the loss of sulfide in our primary standards by either adsorption or oxidation due to trace amounts of oxygen remaining in the solution or as part of the headspace in the container. For the purposes of calibration, secondary standards were made fresh by diluting 100 μL of the primary standard to 60.0 mL with deoxygenated Nanopure water. Aliquots of the secondary standard necessary to cover the proper range of sulfide concentrations were added to 4.0 mL of the MDR and diluted to 60.0 mL with Nanopure water. Generally, 1 h or more was allotted for proper color development.

In the field, whole water samples were pumped into 60.0-mL polyethylene bottles to which 4.0 mL of the MDR had been added. For filtered samples, in-line Millipore Durapore filters (0.45 μm) were used. Later tests showed that this filter type can absorb low levels of dissolved MBRS, sometimes quantitatively. In future studies, we therefore recommend Nuclepore polycarbonate membranes, which we have found to be nonabsorbing. All samples were stored on ice and brought back to the lab for analysis. We undertook a careful study of methylene blue stability over time and found that it was stable when refrigerated and stored in polyethylene bottles. In general, analysis of field samples took place within 12 h of collection.

TABLE 1. Eluent Gradient for Methylene Blue Separation on a Dionex PCX-500 Column[a]

	time (min)				
	0	2.5	4.0	5.0	9.0
eluent A (90% CH_3CN/10% H_2O), %	30	40	60	60	90
eluent B (95% H_2O/5% CH_3CN), %	58	43	10	10	0
eluent A (1.0 M $NaClO_4$), %	10	15	28	28	8
eluent A (0.6 M $HClO_4$), %	2	2	2	2	2

[a] Gradient of eluents A (90% CH_3CN/10% H_2O) and B (95% H_2O/5% CH_3CN). In the mobile phase, we found perchlorate a suitable counterion for good isolation and separation of the methylene blue. Eluents C and D were 1 M $NaClO_4$ and 600 mM $HClO_4$, respectively.

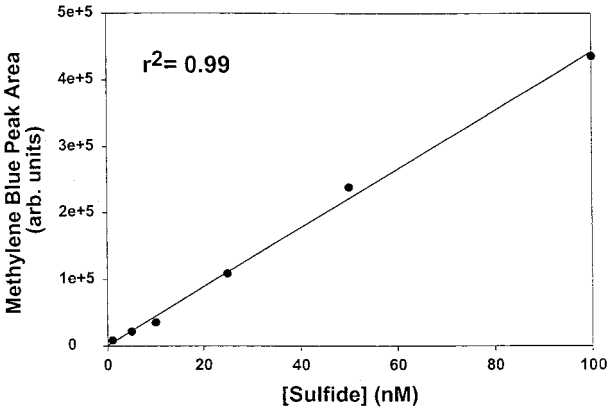

FIGURE 1. Linear relationship of peak area to MBRS concentration was observed throughout the entire range of concentrations (R^2 = 0.997) from 1 to 100 nM MBRS.

For the chromatographic analysis, we use a Dionex DX-500 system with the Dionex PCX-500 column coupled to a Dionex AD 20 absorbance detector. The column packing of the OmniPac PCX-500 consists of a highly cross-linked macroporous core with an ethylvinylbenzene/divinylbenzene internal surface. Polymeric colloids, in the form of latex particles, attach to this core and are functionalized to create acidic sulfonate groups that act as the cation-exchange sites (*17*). Samples were injected into the system using a Dionex autosampler. For controlling the method and the analysis, we used Dionex Peaknet software. For the best separation of the methylene blue, we ran a gradient of eluents A (90% CH_3CN/10% H_2O) and B (95% H_2O/5% CH_3CN). In the mobile phase, we found perchlorate as a suitable counterion for good isolation and separation of the methylene blue. Eluents C (1 M $NaClO_4$, Baker) and D (600 mM $HClO_4$, Baker) establish the appropriate acidic conditions and provide the necessary counterion. Table 1 provides a detailed description of the gradient run for separation of methylene blue on the PCX-500 column. Note, all four eluents are required for the best separation. Injection volumes were 1.0 mL with a flow rate of 1.0 mL/min. The AD 20 lamp setting was set to high, and the optical density was monitored at 666 nm. Peaks resulting from NOM occur much earlier in the chromatogram (2 min−3 min 30 s) than methylene blue (10 min 10 s ± 30 s) under our experimental conditions.

Results and Discussion

To properly calibrate this method, we prepared solutions for analysis ranging from 1 to 100 nM MBRS. A linear relationship of peak area to MBRS concentration was observed throughout the entire range of concentrations (R^2 = 0.997) and is shown in Figure 1. Blanks were run often to ensure that there was no memory of methylene blue from a previous sample. Although we were unable to observe methylene blue peaks

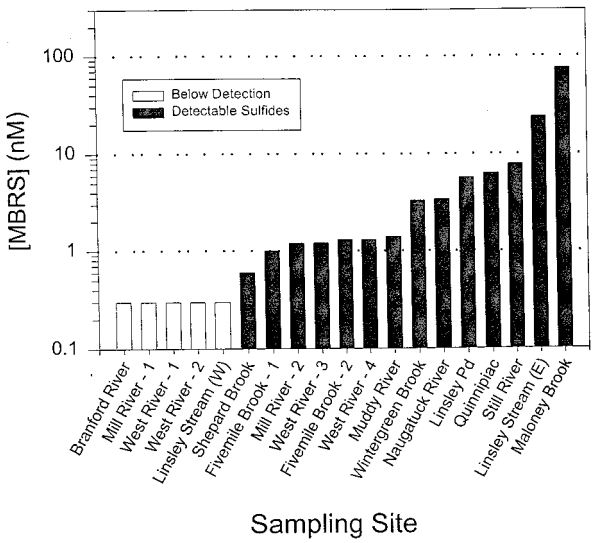

FIGURE 2. Regional stream survey of MBRS. In some instances samples were taken from a variety of locations on same river (e.g., four sites along the West River). These reported values are for whole water samples. All measurements are reported ±3%.

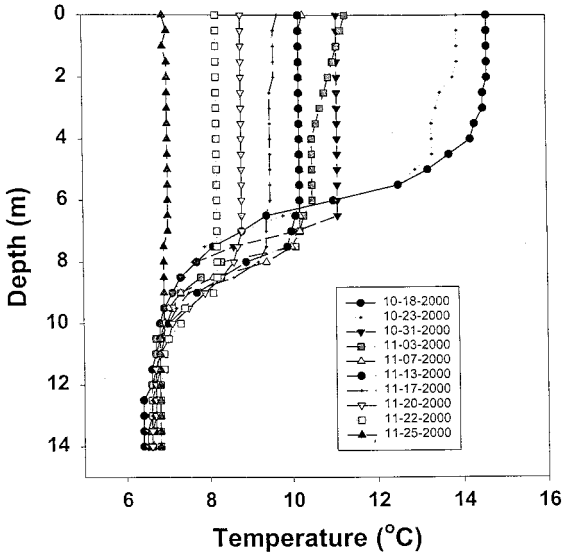

FIGURE 3. Series of temperature profiles taken from the center of Linsley Pond beginning on October 18, 2000. Evidence of the thickening epilimnion is clear from the isothermal layer extending to ca. 8 m by November 20. The pond was isothermal by November 25, 2000.

in our calibration blanks, we calculate the lower limit of detection using this method to be ca. 0.3 nM, based on the standard deviation of 5.3% from our lowest standard, and the normal IUPAC definition (viz., 3× the standard deviation of the lowest measurement). On the basis of uncertainties associated with the linear regression as determined by the method of least squares, we report MBRS concentrations to within ca. ±3%.

On the basis of several duplicate and triplicate measurements of samples of Linsley Pond surface water, we find an average standard deviation of 0.24 nM. This is for repeated *samples* and includes uncertainty introduced by all steps in the process (sampling, transport, storage, analysis). Our laboratory analytical uncertainty is much lower (0.1 nM) for repeated measurements of a standard containing 5.0 nM.

New Haven Region Streams. Our ultimate goal is to identify and quantify specific reduced sulfur compounds by chromatographic and electrochemical methods. To aid in

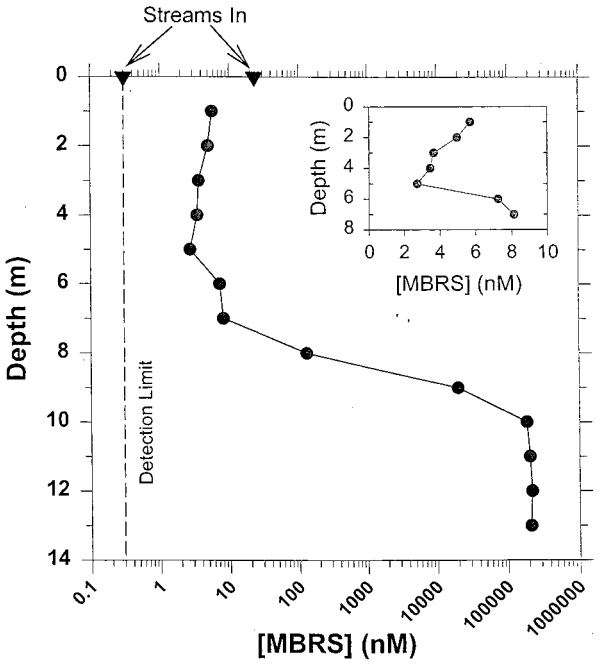

FIGURE 4. Concentration of MBRS in whole water samples from Linsley Pond with depth. Samples were taken at 1-m increments. To better represent the large differences in MBRS concentration between the eplimnion and the hypolimnion, the MBRS concentrations are plotted on a log scale. The dotted vertical line is the detection limit. The inset shows the absolute concentrations of MBRS from 0 to 7 m. MBRS concentrations may be greater near the surface as one of the two streams entering Linsley Pond (Figure 2) show relatively high levels of MBRS.

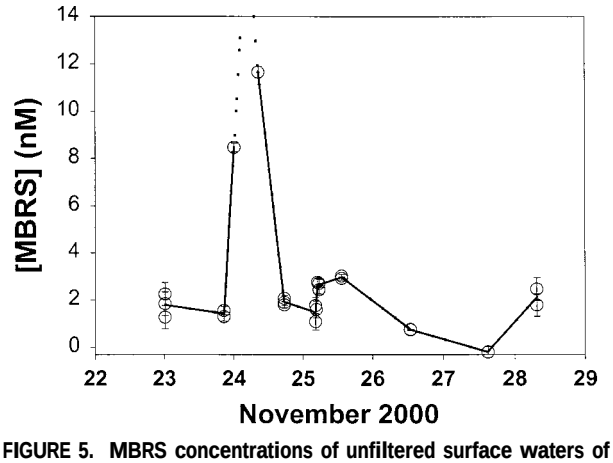

FIGURE 5. MBRS concentrations of unfiltered surface waters of Linsley Pond during 5 days in November 2000. Theses measurements were made by the classical methylene blue method couple to HPLC. On the basis of a number of replicates from each sampling event, random errors associated with sampling never exceeded ±4%. This value is represented by the error bars above for each day. The dotted lines extending upward indicate the potential spike in MBRS that was most likely missed during sampling.

site selection for that research, we conducted a reconnaissance of regional streams and rivers. MBRS levels ranged from less than 0.3 to 74 nM in the 14 water bodies tested so far (Figure 2). In some cases, MBRS were measurable in both whole and filtered waters. However, the MBRS measurements reported in Figure 2 are from unfiltered samples. As mentioned previously, our inability to measure MBRS from filtered waters was due to strong MBRS sorption to the filters we were using. Other trends or patterns are yet to become evident; however, it is not only polluted sites that tend to be higher in MBRS. For example, the three highest and five of

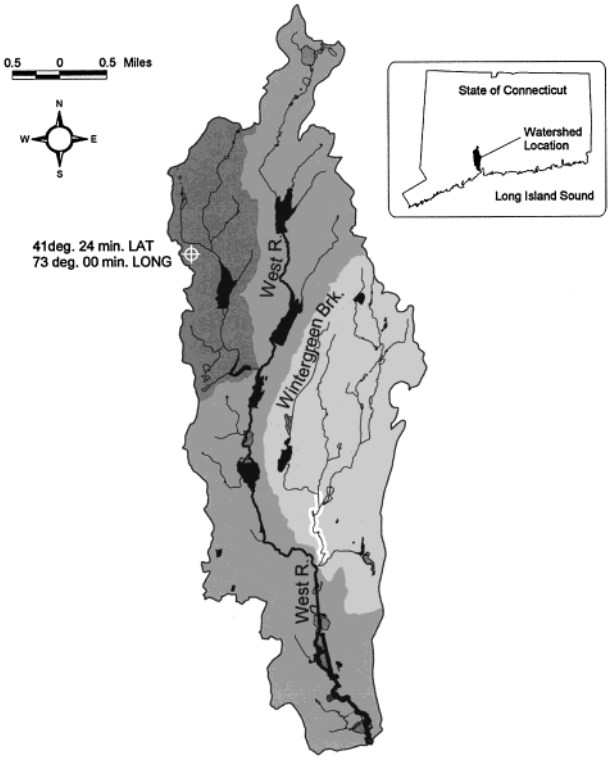

FIGURE 6. Area map of the West River Watershed. The portion of Wintergreen Brook where MBRS were measured is highlighted in white.

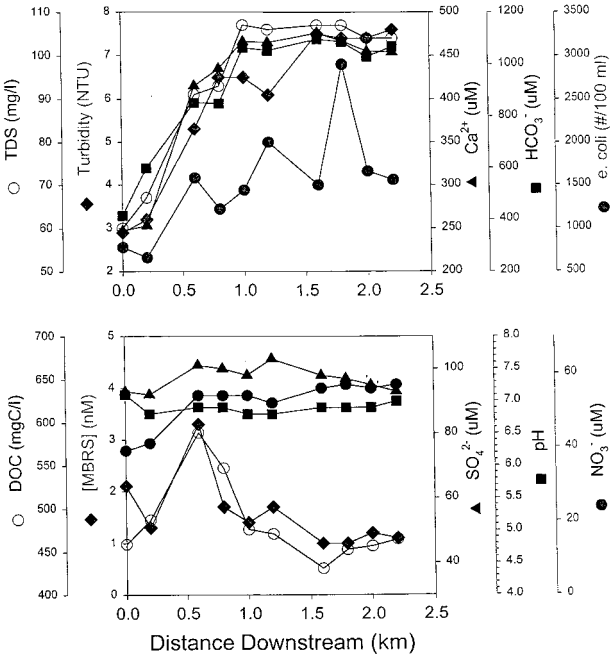

FIGURE 7. Comparison of dissolved sulfides to some of other components measured in this survey. Each point is sampling station. The sulfide pattern exhibited is similar to that of pH, DOC, nitrate, and sulfate, which remain nearly constant downstream. Other components measured in this survey (total dissolved solids (TDS), turbidity, calcium, bicarbonate and *E. coli*) show an increase in concentration downstream.

the highest eight measurements were from sites receiving only low levels of nonpoint source pollution.

Linsley Pond. Linsley Pond is located in the towns of Branford and North Branford, CT. The pond has a surface area of 9.4 ha and a maximum depth of 14 m. The watershed of 236 ha includes mainly low-density residential development. The pond has been studied extensively by Hutchinson and colleagues (*18*) and is perhaps the most thoroughly investigated lake of its size in the world.

In the summer months, this pond becomes thermally stratified and remains that way well into the fall. A series of temperature profiles in October−November 2000 reveal the evolution of stratification (Figure 3). On October 18, there was a thin epilimnion (0−4 m) and thick hypolimnion (8−14 m). The distinctive smell from samples of lake bottom water indicated high concentrations of MBRS (measured to be ca. 200 μM). As the season progressed and the surface water cooled, the thermocline eroded and the epilimnion thickened. In some instances, stratified lakes experience a sudden overturn or mixing when the epilimnion becomes isothermal with the hypolimnion. Our hope was to measure the concentration of MBRS in Linsley Pond surface waters at the time of the overturn and for some hours thereafter.

Lab experiments indicated a half-life with respect to oxidation of the bottom-water sulfide near 23 min. (MBRS are apparently almost entirely H_2S and HS^- in the hypolimnion of this lake.) Thus, it should take approximately 7 h for sulfides to decline 6 orders of magnitude (2^{20}) and fall below our detection limit. We expected to see a spike in MBRS concentrations followed by their rapid decline as oxidation took its course.

MBRS concentrations were measured at 1-m increments on November 20, when the epilimnion had thickened to 8 m (Figure 4). To more clearly represent the large differences in MBRS concentration between the epilimnion and the hypolimnion, the MBRS concentrations are plotted on a log scale. Interestingly, MBRS concentrations were below even our detection limits for *filtered* waters from 0 to 7 m depth (not shown). Again, we explain this by simple sorption of the MBRS to the Millipore Durapore filters. Having switched to Nuclepore filters in more recent experiments, this artifact disappears, and it appears that virtually all of the MBRS we measure are dissolved (or colloidal).

The erosion of the epilimnion continued gradually, and as the days became much cooler, MBRS were measured several times each day. Figure 5 shows MBRS concentrations for whole surface waters over the course of 6 days when the lake became isothermal. The gradual mixing of the lake precluded a large spike of sulfide concentrations; however, a spike about 10 times normal in MBRS concentrations was observed on Thanksgiving (November 25) in two consecutive collections several hours apart. Because of the rapid oxidation and coarse sampling interval, it is possible that an even higher spike may have been missed, as is suggested by the dotted line. Observing a spike in MBRS at such a low concentration is only possible through coupling the classical methylene blue method to HPLC.

After mixing, surface water MBRS returned to values near 2 nM. We believe that the source of these compounds is a brook entering the lake having an MBRS level of 24 nM (see below) rather than bottom-water sulfides, which are probably completely oxidized in a short time.

Wintergreen Brook. During a water chemistry survey of the West River Watershed, New Haven County, CT (Figure 6), MBRS were measured along Wintergreen Brook, which is a small tributary to the West River. The purpose of the survey was to evaluate nonpoint sources of pollution. No industrial point sources occur over the survey region of this watershed, which has an area of 9065 ha. Samples were collected during the descending limb of a storm hydrograph on September 27, 2000, roughly one day after a 1.3-cm rain event. A broad range of water quality parameters were measured, and MBRS were added to this list. Ten samples were collected roughly every 0.2 km over a stream reach

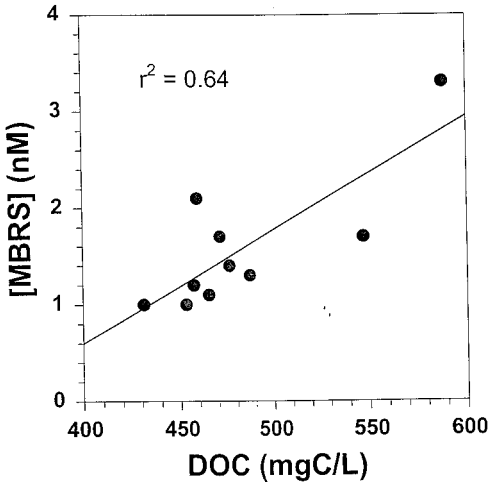

FIGURE 8. Correlation of MBRS with DOC in Wintergreen Brook on September 27, 2000. The best fit line is included. $R^2 = 0.64$; $P = 0.03$.

where several tributaries enter and the brook passes near a closed landfill between stations 8 and 9.

Moving downstream, water quality parameters exhibit one of two distinctive patterns (Figure 7). Major ions (Na^+, Ca^{2+}, K^+, Mg^{2+}, Cl^-, HCO_3^-), total nitrogen (TN), conductivity, fecal coliform, and suspended matter increase dramatically. Dissolved organic carbon (DOC), NO_3^-, phosphate, pH, and sulfate have nearly constant concentrations or decline with distance downstream. MBRS exhibited a pattern similar to this latter group, and concentrations are in good agreement with reported MBRS in other fully oxygenated systems. In particular, MBRS and DOC were significantly correlated (Figures 7 and 8), raising the possibility that the two are directly associated. The nutrients and DOC are closely linked with biological processes and are most abundant in shallow soil layers. We tentatively hypothesize a soil biological origin for the MBRS at this site, but much additional research will be required to evaluate this idea.

Discussion

The classical methylene blue and Cline methods have been used for measuring a pool of reduced sulfur compounds for decades. Here we have shown that both the sensitivity and reliability of these methods can be increased by coupling them to HPLC. This finally allows for the direct measurement of this fraction of dissolved reduced sulfides in oxic freshwaters. We hope to further exploit this method and observe correlations between MBRS and pollution, aquatic ecosystem type, and local land use.

We also hope to begin to study the partitioning between MBRS and those reduced sulfur compounds that are not acid-labile but require a strong reductant to strip sulfides prior to their acidification and treatment with MDR. In sediments, Cr(II) has commonly been used as this reducing agent (*19*). However, preliminary work with oxic surface waters indicates interference from sulfate reduction by Cr(II) (*20*). Further work in this area is required to develop a simple method for quantifying non-MBRS.

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